Scientific Electronic Archives
Issue ID: Sci. Elec. Arch. Vol. 12 (6)
December 2019
DOI: http://dx.doi.org/10.36560/1262019986
Article link
http://www.seasinop.com.br/revista/index.php?journal=SEA&page=article&op=view&path%5B%5D=986&path%5B%5D=pdf
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Comparison of the growth of promastigotes cellular lineages of *Leishmania amazonensis by* the sequential adaptation of Schneider's insect for RPMI 1640 medium culture.

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Abstract. Leishmaniases are neglected zoonoses transmitted by protozoan parasites belonging to the genus *Leishmania*. Because of this, research is being developed to find new drugs, for effective treatment, making it necessary to use liquid culture media to promote the growth of promastigotes forms of *Leishmania*. We sought to compare cell growth in an initial culture medium of 100% Schneider's Insect to a final culture medium containing 100% RPMI 1640, to reduce the cost of research, since RPMI is more accessible. The parasites were first cultivated in 100% Schneider's Insect medium, with a maximum number of $2.012x10^8$ cells/mL, which were transferred, with an appropriate methodology, to media with lower concentrations of Schneider, with a maximum number of $2.35x10^7$cells/mL for the lower concentration of Schneider. The 50%Schneider/50%RPMI concentration cells showed excellent cell motility and viability; however, in concentrations <50%Schneider, motility was compromised and, in 100%RPMI, the cells entered in the phase of decline. With correct handling and constant repikes, the *Leishmania amazonensis* strains can adapt to the RPMI 1640 culture medium without much interference in its cellular viability, maintaining the morphological characteristics, even after exhaustive nutrient replacements and metabolites removal by centrifugation.
Keywords: Leishmaniasis. Zoonosis. Cell Culture. Neglected Diseases.

Introduction

The leishmaniases are infections/diseases naturally transmissible between men and other kinds of mammals, that is, they're zoonosis. These diseases are caused by protozoan parasites of *Leishmania* genus, besides, according to the species of this genus and the clinical manifestation presented, they can be subdivided into (i) cutaneous/tegumentary leishmaniasis (CL/ TL), (ii) mucocutaneous leishmaniasis, (iii) diffused cutaneous leishmaniasis, (iv) diffuse allergic cutaneous leishmaniasis- extremely rare and severe form-, and (v) visceral leishmaniasis (Camargo; Barcinki, 2003). At least, seven species of *Leishmania* are related to human diseases, extra, in Brazil, *Leishmania (Viannia) braziliensis* e *Leishmania (Leishmania) amazonensis* are the most widely distributed species (Grimaldi Jr, 1987 apud Dorval, 2006).

According to World Health Organization (WHO) calculation, the notification of leishmaniasis is compulsory in 30 countries, considering a total of 88, and, concerning the total of cases already notified about TL, 90% were registered only in six countries, including Brazil (Desjeux; Dedet, 1999). Also occurring in other countries of the New World, American Tegumentary Leishmaniasis is considered a public health problem, since it's epidemic potential disease, with broad geographic distribution, as well as it's possibility of taking forms that can cause destructive, disfiguring and incapacitating lesions, with vast repercussion in the psychosocial field of the individual (Gontijo; Carvalho, 2003).

The most commonly used drugs against leishmaniasis are pentavalent antimonials- used as a first option -, pentamidine and amphotericin B - both as second options. However, some problems diminish the efficacy of these drugs, such as parasite resistance, manifestations of side effects and parenteral administration (Comandolli-Wyrepkowski, 2017).

The insufficiency of the knowledge produced about the mechanism of action of drugs in the body, the composition and structure of them is notorious, even with the use of drugs for at least 50 years. Furthermore, they present cytotoxicity, since antimonials accumulates, in general, in vascularized organs and tissues, principally kidneys and liver, besides having a great affinity for spleen and blood (Rath, 2003). Given the above, there is such a need

for the research and development of new active components, besides the necessary investments for new drugs, resulting in a new row of therapy truly efficient.

In the 20th century, between 1907 and 1912, Harrison and Carrel work out with cell culture techniques to study the behavior of animal eukaryotic cells outside the body. These procedures are still widely used as research tools in laboratories worldwide (Alves; Guimarães, 2010). The *in vitro* cultivation of *Leishmania* species is a proficient methodology to obtain a sufficient number of parasites for better diagnosis and treatment, besides for the understanding of the parasite-host relationship and the characterization of the immuno-biology of the parasite (Santos, 2015).

The culture medium is a qualitative and quantitative association of substances that provide the necessary nutrients for the growth of microorganisms *in vitro*. Due to the wide metabolic diversity of organisms, there are several types of medium, which can be classified according to their physical state: (i) liquids: indiscriminate growth with turbidity of the medium; (ii) solids: growth of isolated colonies, widely used for pure cultures; and (iii) semi-solids: there is the addition of less agar and allows bacterial mobility (Nascimento; 2010).

Liquid medium are commonly used for *Leishmania*'s culture, as semi-solid media require further manipulation (Grekov et al*.,* 2011). In addition, microorganisms are rigorous when it comes to the nutrients to be added to the culture medium, since some components, or different batches of the same medium, can be toxic, impairing the cell growth of the organism in question. In the culture of *Leishmania* spp., the mostly medium used are 199H, RPMI 1640, Schneider's Insect, Dulbecco, Grace, among others (Chance et al*.,* 1974; Hendricks et al., 1978; Childs et al., 1979, apud Santos, 2015).

Regularly, different types of culture medium, with different supplementation - such as sterilized human urine or Fetal Bovine Serum (FBS)-, are used according to *Leishmania* sp., with a view to maintain the same intrinsic and extrinsic characteristics of the parasites (Santos, 2015). Though these methodologies have an expensive, the adaptation of cell lineages to cheaper culture medium and forms of supplementation have being stead researched.

The Schneider's Insect medium, produced for insect cells, is quite expensive and used on the culture of *Leishmania* spp., since it has, in large quantities, the necessary nutrients for cell maintenance, thus having a qualitative and quantitative aspect. If supplemented, increases the capacity of cell reproduction and assists in the establishment of cultures. On the other hand, RPMI 1640, if correctly supplemented, can also be used in the culture of *Leishmania* spp.

With the adaptation of fastidious lineages of *Leishmania* spp. for culture medium RPMI 1640, it is possible to reduce the cost of research and to compare the nutritional plenty both medium offer, evaluating, as primer point, the cell viability (morphology, motility, reproduction). However, administrate cell culture is arduous, once the adaptation of different parasites may differ, even if they belong to the same species (Dorta et al., 2012). But, even there, it is necessary for experiments with high-grade performance and low cost.

Therefore, this research was conducted to evaluate the performance and adaptation of different culture medium in a graduated way for the culture of *Leishmania amazonensis.*

Methods

Lineages of Leishmania amazonensis

The promastigotes forms of *L. amazonensis* (WHOM/BR/76/Josefa), which were kindly provided by professor Alexandre Barbosa Reis, from the Federal University of Ouro Preto, were thawed, centrifuged- with RPMI 1640 to remove the glycerol- and cultivated.

Culture of Leishmania amazonensis

The culture medium was made in a 25 cm^3 culture bottle with a cover and filter, culture medium was first composed of Schneider's Insect supplemented with 10% FBS and 3% of human urine, during five days, respecting the period of reactivation of cellular activity, which, in this case, was four days. At each five-day interval, the cells were centrifuged, resuspended and transferred to a new culture bottle, which would always contain less Schneider's Insect than the RPMI 1640 medium

Cellular quantification of Leishmania amazonensis

Direct counting was performed using the Neubauer chamber method, in which the cells are individualized. This chamber is a glass slide with divisions that form nine squares of $1mm^2$, which facilitate the counting. For the quantification, only the four external squares were used.

Medium conditions

The first concentration made for the medium was composed of 100% Schneider's Insect, 10% FBS and 3% sterile human urine, and the last concentration was composed of 100% RPMI 1640 + 10% FBS and 3% sterile human urine (Figure 1). The repikes were made each five days and the concentration of Schneider's Insect was gradually reduced.

Comparison of medium composition

The compositions of the culture medium were obtained according to the information provided by the supplier. The comparisons were made by evaluations of the nutrients present and the different volumes of them.

Results and discussion

The adaptation of *Leishmania amazonensis* parasites to different medium concentrations of

RPMI 1640 and Schneider's Insect was analyzed by cell quantification and growth curve (Figure 2). After the respected period of four days- to return to post-thaw cell viability-, the first repike and the first culture of *Leishmania* sp. were made in a medium (1) containing 100% Schneider's Insect, supplemented with 10% FBS and 3% sterile human urine.

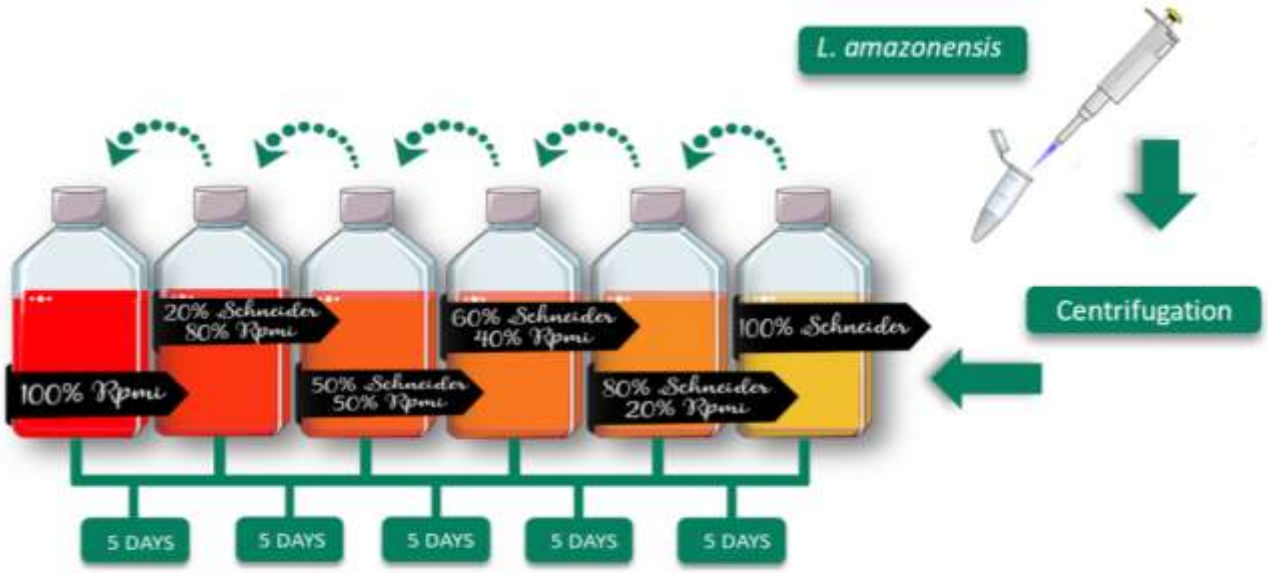


Figure 1. Representative scheme of the sequential adaptation of *Leishmania amazonensis* from Schneider's Insect to RPMI 1640. The lineage of the parasites was first centrifugated and cultivated in a medium with 100% of Schneider's Insect medium for five days, after this, the cells were transferred, with appropriate methodology and five-days permanence in each concentration, to 80%Schneider's/20%RPMI, 60%Schneider's/40%RPMI, 50%Schneider's/50%RPMI, 20%Schneider's/80%RPMI and to 100%RPMI.

The parasites of these culture flasks showed a maximum growth after five days of, on average, $2.01*10^8$ cells/mL (Table 1). Not far away, the same was also observed for parasites that were transferred from medium 1 to medium 2- which contained 80% of Schneider's Insect and 20% of RPMI 1640, following the same supplementation described previously-, the maximum number being $2.00*10^8$.

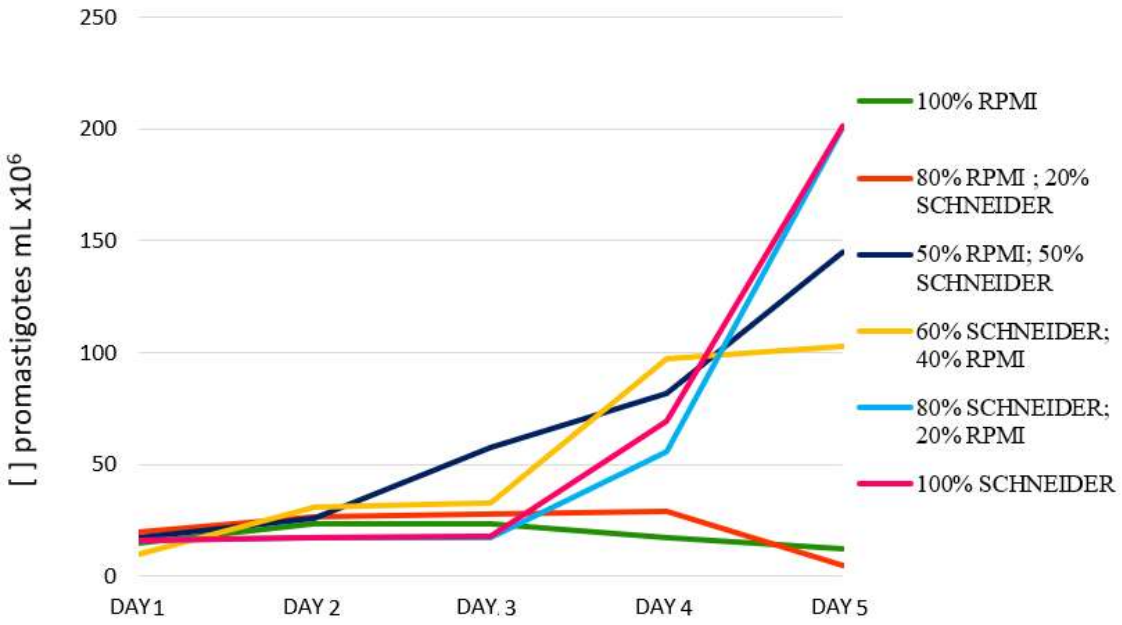


Figure 2: Growth curve of the adaptation, in percentage, of "Leishmania amazonensis" parasites to the sequential transition of promastigotes from Schneider's Insect to RPMI 1640. The graph represents the growth curve of the promastigotes forms of Leishmania amazonensis. The lines indicate the growth of protozoans in different medium concentrations over five days.

Days	***100% Rpmi***	***80% Rpmi; 20% Schneider's***	***50% Rpmi; 50% Schneider's***	***40%Rpmi; 60% Schneider's***	***20% Rpmi; 80% Schneider's***	***100% Schneider's***
Day 1	$1,5*10^7$ cells/mL	$1,99*10^7$ cells/mL	$1,69*10^7$ cells/mL	$9,6*10^6$ cells/mL	$1,59*10^7$ cells/mL	$1,58*10^7$ cells/mL
Day 2	$2,32*10^7$ cells/mL	$2,62*10^7$ cells/mL	$2,6O*10^7$ cells/mL	$3,08*10^7$ cells/mL	$1,69*10^7$ cells/mL	$1,72*10^7$ cells/mL
Day 3	$2,35*10^7$ cells/mL	$2,76*10^7$ cells/mL	$5,75*10^7$ cells/mL	$3,28*10^7$ cells/mL	$1,70*10^7$ cells/mL	$1,76*10^7$ cells/mL
Day 4	$1,71*10^7$ cells/mL	$2,88*10^7$ cells/mL	$8,18*10^7$ cells/mL	$9,74*10^7$ cells/mL	$5,59*10^7$ cells/mL	$6,91*10^7$ cells/mL
Day 5	$1,23*10^7$ cells/mL	$4,63*10^7$ cells/mL	$1,45*10^8$ cells/mL	$1,03*10^8$ cells/mL	$2,00*10^8$ cells/mL	$2,01*10^8$ cells/mL

Table 1: Cellular quantification of the different culture media in the five days of cell culture/mL.

In medium (3) containing 60% Schneider's Insect and 40% RPMI and in medium (4) with 50% Schneider's Insect and 50% RPMI - both following the same supplementation protocol -, the highest number of parasites was around $1.24*10^8$, half of the number found for medium 1 and 2, but, despite the current situation, cellular viability and even cellular morphology were very similar (Figure 3; Figure 4 A-B).

The number of parasites from media (5 and 6) of lower concentration for Schneider's Insect was less than $4.63*10^7$ cells/mL during all experimental stages. On the other hand, the cells of mediums 1-4 showed good metabolic activity, whereby mediums 1 and 2; 3 and 4 showed similarities in results- in terms of the number of protozoans per mL-, and the cells of mediums 1 and 4 seem to have similar morphology. Considering the accompaniment made of the growth curve of the parasites of *Leishmania amazonensis* and it's results, it can be inferred that this method is practical to analyze the cellular growth, since, with the counting of the cells per mL, it is possible to evaluate if the culture medium, chosen by the researcher, is being able to maintain a sufficient production of parasites.

By using supplementary methods, greater success in the cultivation of the parasites is allowed, measured by the cellular quantification, emphasizing the importance of evaluating this variable throughout the experimental period. Interpreting Figure 2, it is possible to infer that, at least, two or three days of culture are required to analyze if the medium culture can provide better metabolic activity and better assistance to support a greater number of repikes.

In the literature, there are few studies on how to effectively select the best culture medium to maintain the parasites and, in the majority of cases, the impression that the parasites are not establishing themselves adequately, only occurs after a considerable time of repike, where the behavioral characteristics of the culture are no longer the same as observed in other studies.

The growth pattern of *Leishmania amazonensis*, observed in culture for 5 days, presented variations according to the medium, in which, when proliferation occurred, it happened in an intense way and in different periods, which, in some cases, the logarithmic and stationary phases take longer to occur.

In an *in vitro* characterization study of *Leishmania braziliensis* species with exposure to different culture medium, Fernandes (2016) recognized excessive proliferative activity on the sixth day of culture, while, here, there is intense proliferative activity for the medium, with concentrations equal to or higher than 50% Schneider's Insect, on the fifth day.

As described by Lemesre (1988), parasites of the species *Leishmania (V.) braziliensis* do not show good growth in the medium RPMI 1640 and the initial concentration of parasites directly influences the growth curve, where the lower the density of parasites in the initial culture medium, the longer the delay in the logarithmic phase- if present. This fact occurs because the parasites need a minimum quantity of some metabolites produced by themselves.

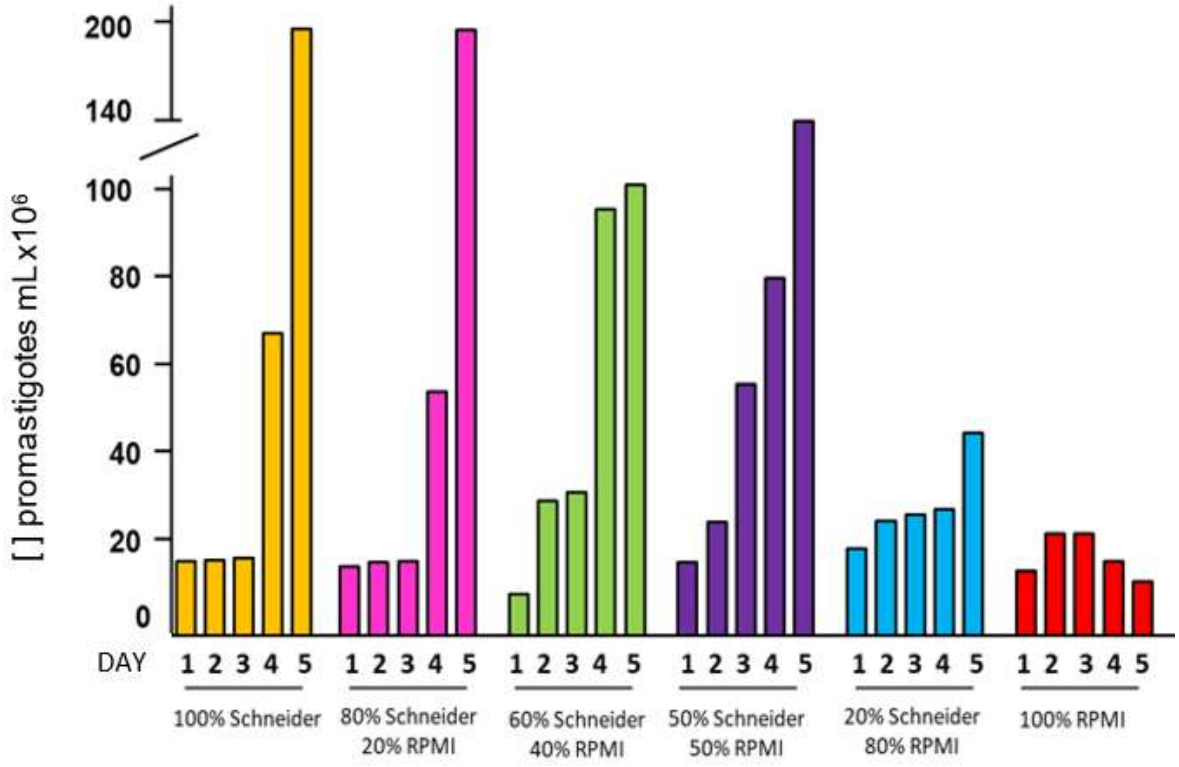


Figure 3. Cellular quantification of Leishmania amazonensis**.** The graphs represents the growth of *Leishmania*'s promastigotes cells- which were quantified all five days of adaptation to the different culture mediums.

Previous studies have described that parasites of the Trypanosomatidae family consume glucose as an energy source, generating the production of organic acids, favoring the reduction in the pH of cultures, a factor responsible for the metacyclogenesis of parasites (Bates; Tetley, 1993; Van Hellemond et al., 1998).

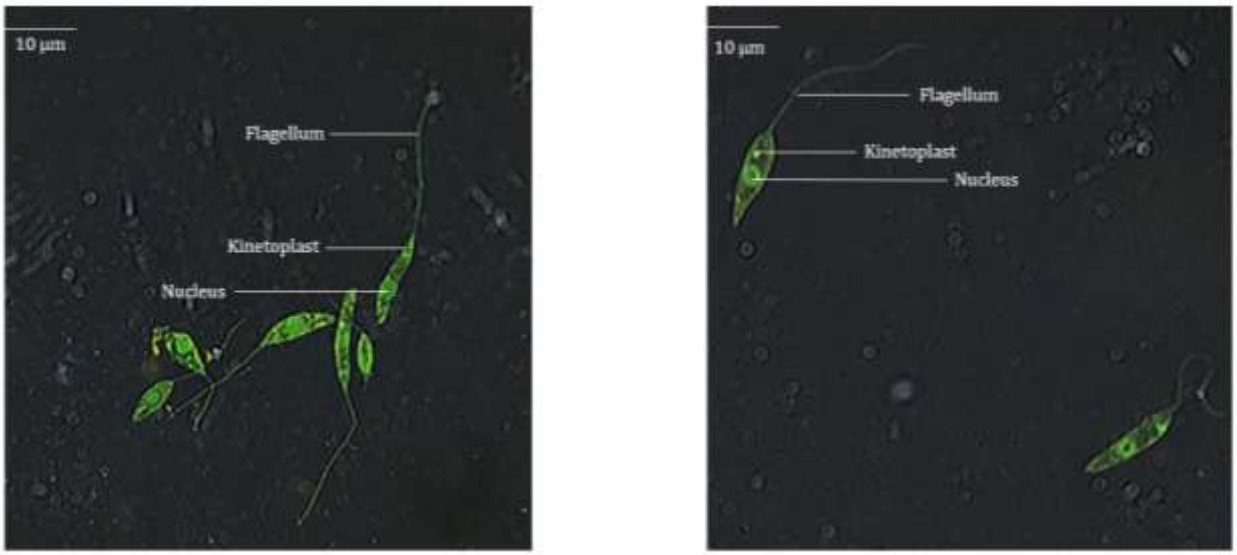
The RPMI 1640 culture medium, for being more qualitative than quantitative, has a lower quantity of glucose in detriment to Schneider's Insect, a factor that may be responsible for the induction of early metacyclogenesis, damaging the proliferation of parasites and resulting in a lower cellular quantification.

Another factor to be considered is simply the insufficient quantity of nutrients present in the medium RPMI 1640, where the parasites don't have enough energy to start the mitotic cycle, resulting in low parasitic density and accelerated cell death.

Cells cultivated in culture medium composed of 50% RPMI 1640 and 50% Schneider's Insect, with appropriate supplementation, showed growth and morphology that should be considered to select the best cost-benefit for the cultivation of these protozoa.

Depending on the cell lineage, supplementation factors can still be replaced, such as the replacement of Fetal Bovine Serum- very expensive- for enrichment with high concentrations of amino acids, such as sterile human urine (Howard et al. 1991, Armstrong; Patterson, 1994).

Therefore, with the correct handling and constant repikes, *Leishmania amazonensis* is capable of adapting the culture medium without much interference in its cellular viability, maintaining the morphological characteristics, even after the exhaustive replacement of nutrients and removal of metabolites by centrifugation.



(A) (B)

Figure 4. Optical microscopy (400x) of promastigotes forms of *Leishmania amazonensis* cultivated in culture medium containing (A) 100% Schneider's Insect and (B) 50% Schneider's Insect and 50% RPMI 1640, both containing 3% sterile human urine supplementation and 10% Fetal Bovine Serum.

Conclusion

The analysis of the growth curve of cells assists in determining which medium to use to maintain a culture with sufficient numbers of parasites.

Leishmania amazonensis is capable of maintaining the morphological characteristics on RPMI 1640 medium with the correct handling, constant repikes and supplementation.

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